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293 Cell Lines That Inducibly Express High Levels of Adenovirus

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293 cell lines that inducibly express high levels of adenovirus type 5 precursor terminal protein (pTP) under the control of a tetracycline-dependent promoter were constructed. To construct the cell lines expressing pTP, 293 cells were stably transfected with a plasmid encoding the tetracycline repressor/VP16 transactivator protein (tTA) using selection with hygromycin. Cell lines that expressed high levels of tTA activity were then stably transfected with plasmids in which pTP expression is directed by the tTA-dependent promoter from either a cDNA or a modified genomic construct using selection with G418. Cell lines that expressed high, inducible levels of pTP efficiently complemented a temperature-sensitive pTP mutant virus for growth and plaque formation at the nonpermissive temperature.

INTRODUCTION

Replication of the linear, 36-kb adenovirus chromosome occurs by leading strand only (reviewed in Hay *et al.,* 1995; Kelly, 1984; Stillman *et al.,* 1981; Van der Vliet, 1995). Replication is dependent on expression of the products of the E2 region, including the single-strand DNA binding protein, encoded by E2A and required for binding of the large amount of single-stranded DNA produced, and the precursor terminal protein (pTP), which acts to prime replication, and the virally encoded DNA polymerase (DNA pol), which are encoded by E2B. The mRNAs encoding pTP and DNA pol are derived by differential splicing. Translation of both proteins is initiated by synthesis of a tripeptide encoded by the small exon from 39 map units (Shu *et al.,* 1988). The coding sequences then diverge at the main exons. The coding regions for the 5′ end of DNA pol and the 3′ end of pTP overlap. In addition to these two well-characterized products, the primary transcript is also spliced to an acceptor that lies in the middle of the region encoding the main exon of pTP (Broker *et al.,* 1984). The role of this mRNA and the putative protein that it encodes is not clear.

Construction of replication-incompetent adenoviruses offers potential for understanding the role of pTP in the infectious cycle. In addition, replication-incompetent viruses offer promise as gene therapy vectors. The adenovirus vectors that are currently in use consist primarily of viruses that are replication-defective and, thus, replicate to low levels in the absence of complementation. This low level of replication appears to contribute to an inflammatory response that leads to destruction of transduced cells *in vivo* (Engelhardt *et al.,* 1994a,b). Deletion of any of the viral gene products encoded by E2 should lead to inability of the virus to replicate in the absence of complementation. Viruses deleted for the E2A gene have been constructed (Rice and Klessig, 1985) and grown in cells that provide E2A in *trans* (Klessig *et al.,* 1984a,b; Rice and Klessig, 1985; Brough *et al.,* 1992). These viruses are replication incompetent. However, the E2A protein is required in very large amounts during the infectious cycle, and thus, E2A deletion mutants are relatively difficult to efficiently complement. In contrast, pTP and DNA pol are required in substantially lower amounts. Thus, pTP or DNA pol deletion mutant viruses may be more readily complemented by cell lines that express the appropriate gene product.

Attempts to make cell lines expressing pTP proved very difficult, with suggestive evidence that expression of pTP may block cell cycle progression. However, cell lines that constitutively express pTP have been constructed (Schaack *et al.,* 1995a). While these cell lines complement a temperature-sensitive pTP mutant virus for growth at the nonpermissive temperature, cell lines that inducibly express higher levels of pTP should permit more efficient complementation of viruses lacking pTP expression and will permit the role of pTP within the cell to be more readily studied.

Many inducible promoters in mammalian cells have been described. The majority of these promoters are con-

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trolled through inhibition of transcription by a steric blocking agent or in a positive fashion by an inducer. The process of induction may have substantial effects on cellular processes other than that of expression of the gene of interest. The inducible promoter system of Gossen and Bujard (1992), in contrast, relies on the use of a simple basal promoter whose expression is positively dependent on an artificial transcription factor introduced into the cells. The simple basal promoter has multiple tetracycline operators introduced upstream of the basal promoter. The activity of this promoter is dependent on a fusion protein consisting of the tetracycline repressor and the activating domain of the strong herpes simplex virus (HSV) transcriptional activator VP16 (this protein is called tTA). The tetracycline repressor binds to the tetracycline operator with much higher affinity in the absence than in the presence of tetracycline. Thus, cells grown in the presence of tetracycline express the introduced gene at very low levels, while removal of the tetracycline leads to substantial activation at the level of transcription. We have made use of this system to inducibly express high levels of biologically active pTP in 293 cells.

MATERIALS AND METHODS

Cell lines

293 cells are human embryonic kidney cells that are transformed by and express high levels of the E1A and E1B proteins of adenovirus type 5 (Graham *et al.,* 1977). 293-pTP_2 and 293-pTP_{13} cells (Schaack *et al.,* 1995a) were constructed by stable introduction of a pTP cDNA behind the artificial tetracycline operator containing minimal promoter (Gossen and Bujard, 1992).

Plasmids

Plasmids used in this study are based upon the plasmids 10-3 and 15-1 (Gossen and Bujard, 1992). The plasmid 10-3 contains a minimal promoter consisting of multiple copies of the tetracycline operator and a TATA box followed by a minimal multiple cloning site. Plasmid 10-3 was modified to 10-3_{MCS} by introduction of a multiple cloning site containing sites for *Sac*II, *Bgl*II, *Xba*I, *Hin*dIII, *Sma*I, *Sph*I, *Eco*RI, and *Bam*HI by hybridizing and ligating the oligonucleotides 5′-GGAGATCTAGAAGCTTCCCGGGCATGCGAATTCG-3′ and 5′-GATCCGAATTCGCATGCCCGGGAAGCTTAGATCTCCGC-3′ between the *Sac*II and the *Bam*HI sites of 10-3. 10-3_{MCS} was then modified by introduction of a neomycin phosphotransferase (neo) gene under the control of the HSV thymidine kinase (tk) promoter. A plasmid containing the HSV tk promoter-driven neo gene, pREP9 (Invitrogen; a gift from J. Corsini), was altered by deletion of the *Hin*dIII–*Xba*I fragment. This deletion removed an *Nru*I site. The resultant plasmid was digested with *Nru*I and ligated with a *Sal*I linker. The neo gene was modified by insertion of an *Apa*I adaptor in the *Eco*RI site within the tk promoter resulting in the addition of 10 bp such that upstream transcriptional elements (Coen *et al.,* 1986) would remain on the same face of the DNA. The neo gene was removed by digestion with *Sal*I and inserted into the *Xho*I site of 10-3_{MCS}. Plasmids were isolated with the neo gene in either the same or the opposite orientation as the tetracycline operator-driven transcription unit. The plasmid in which the two transcription units were in opposite orientations, termed 10-3_{neo}, was used for further plasmid constructions.

The plasmid ppTP-neo, which contains a pTP cDNA under the control of the tetracycline-dependent promoter (Schaack *et al.,* 1995a), was used in the construction of cell lines where pTP was to be expressed from a cDNA. In addition, a modified genomic construct encoding pTP and DNA pol was prepared. The fragment from the *Xho*I site at 10,295 bp to the *Xmn*I site at 14,562 bp encoding the first three amino acids of pTP and DNA pol within the 39-map-unit exon was cloned into pGem2 (Promega). The plasmid was digested with *Bst*EII + *Not*I, blunt ended using the Klenow fragment of DNA polymerase I in the presence of dNTPs, and ligated to delete the L1 coding sequence, the VAII gene, and the majority of the VAI gene contained within the fragment from 10,719 to 13,436 bp. To reduce the size of the untranslated leader, the fragment from the *Kpn*I site at 14,291 bp to the *Bam*HI site within the polylinker was deleted by restriction digestion followed by blunt ending by the Klenow fragment of DNA polymerase in the presence of dNTPs and ligation (this recreates the *Bam*HI site). The modified genomic fragment containing the coding region for the first three amino acids of pTP and DNA pol was transferred to 10-3_{neo} (in which the *Bgl*II site within the multiple cloning site had been modified to a *Bam*HI site) as a *Bam*HI–*Xba*I (bp 10,590) fragment. The resultant plasmid was designated 10-3-pTP/Pol leader. The pTP and DNA pol genes were transferred to 10-3-pTP/Pol leader as an *Xba*I–*Eco*RI fragment (bp 10590–5192) in which the *Eco*RI site had been engineered at the 3′ end of the DNA pol gene by PCR-mediated site-directed mutagenesis using the primer 5′-GGCATGGATCCGTCGACTCGAATTCGCTACGGCATGCCGATCCAGCATA-3′ to generate the plasmid 10-3-pTP/Pol encoding wild-type pTP and DNA pol with the third amino acid from the C-terminus (codon from the template strand underlined) mutated from Gly to Glu.

The plasmid 15-1, containing the tTA gene driven by the CMV promoter, was modified by introduction of a gene encoding resistance to hygromycin. A 2.25-kb *Sal*I fragment from pRV1hygro (a gift from M. Ostrowski) containing the SV40 early promoter, the hygromycin resistance gene, and the 3′ MSV LTR (to provide a polyadenylation signal) was inserted into the *Xho*I site of 15-1, and clones containing the hygromycin-resistance cassette in both orientations were recovered. A clone containing the

SV40 promoter oriented away from the CMV promoter driving expression of tTA was referred to as 15-$1_{hygro(-)}$ and was used to establish tTA-expressing cell lines.

Construction of new cell lines

293 cells were stably transfected with 15-$1_{hygro(-)}$ using calcium phosphate precipitation. Cells were selected in the presence of 50 μg/ml hygromycin and colonies picked and grown in the presence of 25 μg/ml hygromycin.

Determination of tTA activity in cell lines

293-tTA cell lines were transiently transfected in the presence and absence of 5 μg/ml tetracycline with the plasmid 13-3 containing a luciferase gene driven by the tetracycline operator promoter (Gossen and Bujard, 1992), using calcium phosphate precipitation. Forty-eight hours after transfection, the cells were harvested by scraping, pipetted vigorously to lyse the cells, quick frozen, and stored at $-70°$ until use. Luciferase activity was determined in the presence of ATP and luciferin (DeWet *et al.,* 1987).

Detection of pTP in cell lines

Cells were washed with phosphate-buffered saline (PBS), harvested by scraping, lysed in the presence of hypotonic solution (10 m*M* NaCl, 10 m*M* Tris–HCl, pH 8.0, 3 m*M* $MgCl_2$) containing 0.5% Nonidet-P40 and protease inhibitors phenylmethylsulfonyl fluoride, leupeptin, and pepstatin (in other experiments, the presence of protease inhibitors had no effect on pTP stability). The lysed cells were incubated 10 min at 37° in the presence of 20 μg/ml DNase I. The proteins were then denatured, electrophoretically resolved on a 9% polyacrylamide gel (42.5:1 acrylamide:bisacrylamide) containing 0.1% sodium dodecyl sulfate, and transferred to nitrocellulose using a semidry transfer apparatus. The filters were stained with Ponceau S to demonstrate that similar amounts of protein were present from each sample. The blot was treated with blocking solution (1% nonfat dry milk in PBS), incubated with polyclonal antiserum against the C-terminal region of pTP as a fusion protein with β-galactosidase (Schaack *et al.,* 1995a) in blocking solution, washed repeatedly with PBS, incubated with anti-rabbit IgG/alkaline phosphatase conjugate in blocking solution, and washed repeatedly with PBS, and then color reaction was performed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

293-tTA cells

293-tTA cells were constructed by stable introduction of the plasmid 15-1 modified to contain a hygromycin-resistance gene as indicated under Materials and Methods. The tTA activity in the various cloned cell lines was determined in the presence and absence of tetracycline using transfection of the luciferase-encoding plasmid 13-3 (Table 1) (Gossen and Bujard, 1992). Luciferase activity was determined as a measure of tTA activity. The background level of luciferase gene expression in 293 cells and 293-tTA cells in the presence of tetracycline is high, presumably reflecting transcriptional activation of the artificial promoter by E1A protein. Certain of the 293-tTA cell lines exhibited much greater activity in the absence than in the presence of tetracycline. The background activity in the absence of tetracycline was similar to that observed in 293 cells, indicating that tTA binding to the tTA-dependent promoter was inefficient in the presence of tetracycline. Certain of the cell lines exhibited greater that 100-fold induction of luciferase activity in the absence of tetracycline. While this is considerably less than 10^6-fold activation observed in HeLa cells (Gossen and Bujard, 1992), two factors are likely responsible: first, the greatest activity differences observed by Gossen and Bujard were in cell lines stably transfected with both the tTA and the luciferase genes, while in this assay, the cells were transiently transfected with the luciferase-encoding plasmid; second, it is possible that E1A protein activates expression from the tetracycline operator minimal promoter. These points raise the possibility that certain stably transfected 293-tTA cell lines will exhibit greater control of expression than did the transiently transfected cells. 293-tTA_{27} and 293-tTA_{34} cells, which exhibited the greatest level of activation in the absence of tetracycline, were used for subsequent studies.

TABLE 1

tTA Activity in 293-tTA Cell Lines

Cell line	Light units +tet	Light units −tet	Induction
293-tTA_6	1600	1.1×10^5	70
293-tTA_{25}	1700	1.9×10^5	110
293-tTA_{27}	3000	4.2×10^5	140
293-tTA_{31}	2700	3.1×10^5	120
293-tTA_{32}	6200	5.4×10^5	90
293-tTA_{34}	3900	1.9×10^6	490
293-tTA_{35}	1.6×10^4	1.9×10^6	120

Note. Cloned cell lines stably transfected with the tTA gene and grown in the presence of absence of 5 μg/ml tetracycline (tet) were transiently transfected in duplicate with the plasmid 13-3 encoding luciferase under the control of the tetracycline-dependent promoter. Cells were harvested 48 hr after transfection and luciferase activity was determined (DeWet *et al.,* 1987) as indicated under Materials and Methods.

293-pTP cell lines 2 and 13 that were constructed earlier and express relatively high levels of pTP in a tetracycline-independent manner (Schaack *et al.,* 1995a) were tested for tTA activity using transient transfection with the plasmid 13-3 (data not shown). Both cell lines exhibited

background levels of luciferase activity in the presence and absence of tetracycline, indicating that these cell lines do not express tTA. pTP expression in these cell lines is likely due to activation of the tetracycline-dependent promoter by E1A protein (or similar factor) expressed in 293 cells.

Isolation of 293 cell lines that inducibly express pTP

293-tTA cell lines 27 and 34 were stably transfected with the plasmid ppTP-neo, containing the pTP cDNA under the control of the tetracycline-inducible promoter and the neomycin phosphotransferase gene under the control of the SV40 early promoter (Schaack *et al.,* 1995a). Cells were selected in the presence of 400 μg/ml G418 and cell lines were cloned. The cloned cell lines were tested for expression of pTP in the presence and absence of tetracycline (Fig. 1). The analysis demonstrated that a variety of cell lines expressed very high levels of pTP and that pTP expression was inducible in the majority of the cell lines that were positive for pTP. Of the cell lines tested, 293-pTP$_{14}$ cells expressed the highest level of pTP under induction with relatively little pTP expression in the presence of tetracycline. The level of pTP expression in fully induced 293-pTP$_{14}$ cells is sufficiently high that a Ponceau S-stainable band was apparent on the Western filter at the position of pTP prior to immune staining.

Transcripts from the E2B region of adenovirus are differentially spliced to yield mRNAs that encode pTP or DNA polymerase. An additional splice acceptor has been

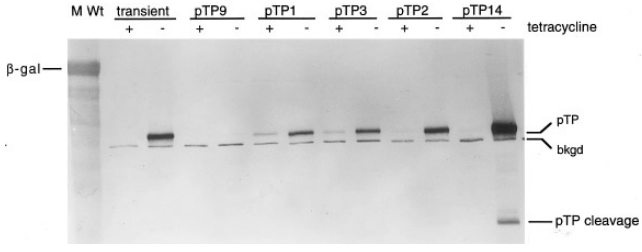

FIG. 1. Western analysis of pTP expression from the pTP cDNA construct in cloned cell lines. 293-tTA clone 34 was transiently transfected (lanes marked transient) and selected 293-tTA clones 27 and 34 were stably transfected (lanes indicated by clone numbers) with the plasmid ppTP$_{neo}$ encoding pTP under the control of the tetracycline-dependent promoter (Schaack *et al.,* 1995a). Cells were grown in the presence or absence of tetracycline. Cell lysates were prepared and proteins from approximately 3×10^4 cells per lane were resolved on a polyacrylamide gel containing sodium dodecyl sulfate and transferred to nitrocellulose. Similar amounts of protein were present in each lane as indicated by staining of the filter with Ponceau S. Western analysis was performed as described under Materials and Methods. The pTP band is indicated along with a background band observed in the absence of pTP and putative proteolytic products of pTP on the right and β-galactosidase, which is recognized by the antiserum, from the marker lane on the left. Clone numbers are listed above the lanes. Clones 9 and 1 were derived from 293-tTA$_{27}$ cells and clones 3, 2, and 14 were derived from 293-tTA$_{34}$ cells.

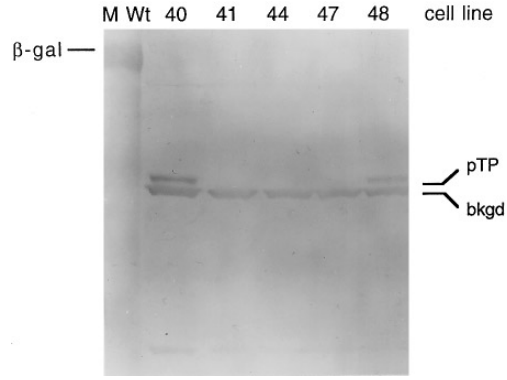

FIG. 2. Western analysis of pTP expression from the genomic pTP/DNA pol construct in cloned cell lines. Cell lines derived from transfection of 293-tTA$_{34}$ cells with 10-3$_{neo}$pTP/Pol and selected in the presence of G418 were grown in the absence of tetracycline. Extracts from approximately 3×10^5 cells per lane were used for Western analysis as described under Materials and Methods. The pTP band is indicated along with a background band observed in the absence of pTP on the right and β-galactosidase, which is recognized by the antiserum, from the marker lane on the left. Clone numbers are listed above the lanes.

mapped roughly to the middle of the region encoding the main exon of pTP (Broker *et al.,* 1984). The function of the alternative pTP mRNA, termed TP* mRNA, is unclear. In an attempt to produce cell lines capable of expressing all three mRNAs encoded by E2B, a modified genomic construct that encodes a primary transcript that has the potential to give rise to all three differentially spliced mRNAs was introduced into 10-3$_{neo}$ to yield 10-3$_{neo}$pTP/Pol (see Materials and Methods). 293-tTA$_{34}$ cells transiently transfected with 10-3$_{neo}$pTP/Pol synthesized pTP as determined by Western analysis. In addition, both pTP and DNA pol mRNAs were synthesized during transient transfection (data not shown). Thus, 10-3$_{neo}$pTP/Pol has the potential to encode both pTP and DNA pol as well as TP*, the putative product of TP* mRNA.

293-tTA$_{34}$ cells were stably transfected with 10-3$_{neo}$pTP/Pol and cells selected and cell lines cloned. Cell lines were tested by Western analysis for ability to synthesize pTP in the absence of tetracycline. Of the 92 cell lines cloned and tested, only 2 were found to express detectable levels of pTP (Fig. 2). This is in striking contrast to the approximately 35% positive rate for cell lines expressing pTP from the pTP cDNA. The low frequency of clones that expressed detectable levels of pTP suggests that expression of DNA pol and/or the TP* product is deleterious to 293 cells. 293-pTP$_{40}$ cells, the line that expressed the highest level of pTP from the modified genomic construct, were analyzed in detail.

The level of pTP expression in 293-pTP$_{14}$ and 293-pTP$_{40}$ cells was compared directly (Fig. 3). The level of expression in the 293-pTP$_{14}$ cells is substantially greater than that in the 293-pTP$_{40}$ cells. Both cell lines exhibited strong inducibility for pTP expression. This analysis demonstrated that both 293-pTP$_{14}$ and 293-pTP$_{40}$ cells ex-

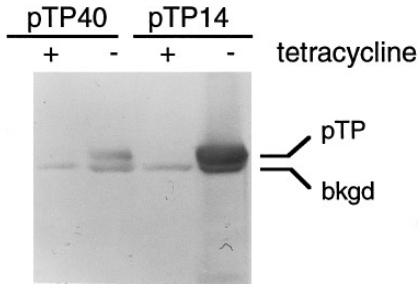

FIG. 3. Comparison of pTP expression levels in 293-pTP$_{14}$ and 293-pTP$_{40}$ cells. 293-pTP$_{14}$ and 293-pTP$_{40}$ cells were grown in the presence and absence of tetracycline. Extracts from approximately 3×10^5 cells per lane were used for Western analysis as described under Materials and Methods. The pTP band is indicated along with a background band observed in the absence of pTP. The cell lines are indicated above the lanes.

press pTP, although TP* was not detected (the smaller products in Fig. 3 are apparent only from the pTP cDNA and appear to be proteolytic products of pTP). However, the polyclonal serum used to detect pTP was raised against a fusion protein containing *Escherichia coli* LacZ and a small portion of pTP beginning at the *Sal*I site at 9842 bp grown in *E. coli* [the fusion protein was proteolyzed to this form during growth of *E. coli* (Schaack *et al.,* 1995a)]. Thus, it is not clear that the antiserum used is capable of recognizing TP*. Polyclonal serum raised against an epitope at the C-terminus of pTP (Fredman and Engler, 1993; a gift from J. Engler) was used to examine the pTP products from both 293-pTP$_{14}$ and 293-pTP$_{40}$ cells (data not shown). Again, the only band apparent from 293-pTP$_{40}$ cells was pTP. Thus, it appears that 293-pTP$_{40}$ cells do not express TP*, at least in the absence of infection.

Changes in pTP expression with passage of cells

Cells were maintained in culture in the presence of tetracycline or doxycycline for 4 months. A modest reduction in the level of pTP expressed after induction in 293-pTP$_{40}$ cells but a greater reduction in the level of pTP expression in induced 293-pTP$_{14}$ cells was apparent relative to low passage cells. Cells passed in the absence of selection for 4 months were found to exhibit greater susceptibility to G418 and hygromycin than were early passage cells. When the cells were returned to growth under selective conditions in the presence of hygromycin and G418, levels of pTP expression returned to that observed in early passage cells (data not shown).

Growth rate of 293-pTP cells

The 293-pTP cell lines constructed earlier through cotransfection of the 10-3-pTP$_{neo}$ and 13-3 plasmids were found to exhibit growth rates that varied inversely with the level of expression of pTP (Schaack *et al.,* 1995a). The cell lines initially formed highly vacuolated giant cells at high frequency. With repeated passage, the cell lines grew more rapidly and exhibited morphology similar to that of the parental 293 cells. This evidence was taken to suggest that pTP may act to inhibit progression through the cell cycle. We examined the rate of growth of 293-pTP$_{14}$ cells in the presence and absence of tetracycline. The cell line generally grew more rapidly in the presence of tetracycline than in its absence. However, three complicating factors were apparent: first, the growth rate of early passage 293-pTP$_{14}$ cells in the presence of tetracycline, where very little pTP was expressed, was greatly reduced relative to parental cells; second, the growth rate varied greatly between passages in a manner that appeared to be dependent, at least in part, on cell density; and third, the parental tTA cells were found to grow more slowly in the presence of tetracycline than in its absence. Thus, it is not clear from these data what role pTP plays in regulating the cell cycle. The pTP-expressing cell lines constructed in this study never exhibited the dramatically altered cell morphology observed with the cell lines constructed earlier, so it is possible that the inducible nature of pTP expression overcame the selective process observed with growth of the cells that constitutively express pTP.

Biological activity of pTP expressed in the cell lines

293-pTP$_{14}$ and 293-pTP$_{40}$ cells were examined for ability to support growth of the temperature-sensitive pTP mutant *sub*100r [modified from the virus *sub*100 (Freimuth and Ginsberg, 1986) by restoration of the E1A and E1B genes to wild-type (Schaack *et al.,* 1995a)] and the

TABLE 2

Plaquing Efficiency of *dl*309 and *sub*100r in 293 and 293-pTP Cells

Virus	Cell line	Temperature	Virus titer
*dl*309	293	32°	1.4×10^8
		39.5°	1.4×10^8
	293-pTP$_{14}$	32°	3.0×10^7
		39.5°	1.6×10^8
	293-pTP$_{40}$	32°	1.4×10^8
		39.5°	3.4×10^8
*sub*100r	293	32°	4.5×10^7
		39.5°	$<10^5$
	293-pTP$_{14}$	32°	4.1×10^7
		39.5°	4.8×10^7
	293-pTP$_{40}$	32°	5.2×10^7
		39.5°	2.1×10^8

Note. Stocks of *dl*309 and *sub*100r grown in 293 cells at 37 and 32°, respectively, were plaque titered on the different cell lines at 32 and 39.5°. Cells incubated at 39.5° were stained on Day 7 and plaques counted on Day 8. Cells incubated at 32° were stained on Day 12 and counted on Day 13. The results from a typical experiment are presented. The plaquing efficiency of *sub*100r in 293 cells was previously shown to be reduced by a factor of greater than 10^4 in 293 cells at 39.5° relative to 32° (Schaack *et al.,* 1995a).

TABLE 3

Yield of *dl*309 and *sub*100r from One-Step Growth Curves in 293 and 293-pTP Cells

Virus	Cell line	Temperature	Virus titer
*dl*309	293	32°	1.2×10^8
		39.5°	1.3×10^8
	293-pTP$_{14}$	32°	4.5×10^7
		39.5°	1.1×10^8
	293-pTP$_{40}$	32°	3.2×10^7
		39.5°	1.0×10^8
*sub*100r	293	32°	3.7×10^7
		39.5°	$<10^5$
	293-pTP$_{14}$	32°	3.3×10^7
		39.5°	3.9×10^7
	293-pTP$_{40}$	32°	4.2×10^7
		39.5°	1.6×10^8

Note. Cells were infected at a multiplicity of 10 plaque forming units per cell. After 2–3 days at 39.5° or 5 days at 32° freeze–thaw lysates were prepared. Virus plaque titers were determined on 293 cells at 32°. The results from a typical experiment are presented. The yield of *sub*100r in 293 cells incubated at 39.5° was previously shown to be reduced by a factor of approximately 600 relative to the yield at 32° (Schaack *et al.,* 1995a).

phenotypically wild-type virus *dl*309 (Jones and Shenk, 1978) at the nonpermissive temperature of 39.5° in plaquing efficiency (Table 2) and one-step growth curve (Table 3) assays. Both cell lines permitted efficient plaque formation and high-titer growth of *sub*100r at 39.5°. 293-pTP$_{40}$ cells actually permitted more efficient plaquing and growth of *sub*100r at 39.5° than at the permissive temperature of 32°. In addition, 293-pTP$_{40}$ cells complemented the *sub*100r defect more efficiently than 293-pTP$_{14}$ cells in spite of the significantly greater amount of pTP present in 293-pTP$_{14}$ cells.

293-pTP$_{40}$ cells were constructed using a modified genomic plasmid that has the potential to encode DNA pol as well as pTP. These cells were examined for ability to complement the temperature-sensitive DNA pol mutant *ts*149 for growth at the nonpermissive temperature of 39.5°. In a one-step growth curve, *ts*149 yield was decreased approximately 7-fold between 32 and 39.5° in 293-pTP$_{40}$ cells while the ratio in parental 293 cells was greater than 30-fold, suggesting that 293-pTP$_{40}$ cells might express biologically active DNA pol. However, the absolute yield of virus in 293-pTP$_{40}$ cells was reduced at both 32 and 39.5° relative to 293 cells. In addition, 293-pTP$_{40}$ cells did not support *ts*149 plaque formation at 39.5°. Examination of mRNAs synthesized after induction of 293-pTP$_{40}$ cells showed that pTP mRNA was synthesized in substantial amounts but that little if any DNA pol mRNA was present (data not shown). The reason for the apparent interference in growth of *ts*149 in one-step growth curves is not clear, but the data indicate that 293-pTP$_{40}$ cells do not efficiently complement the DNA pol mutant *ts*149 for growth at 39.5°.

Transfection of 293-pTP cells

The efficiency with which the 293-pTP$_{14}$ and 293-pTP$_{40}$ cells could be transfected with viral DNA to yield plaques was examined using both *dl*309 and 327$_{Bst}\beta$-gal (Schaack *et al.,* 1995b) DNAs. Parental 293 cells yielded approximately twice as many plaques for both viruses as either 293-pTP$_{14}$ or 293-pTP$_{40}$ cells (data not shown). However, given the extremely high transfection efficiency for the parental 293 cells, the pTP-expressing cells are very efficiently transfected and, thus, will prove useful in the construction of pTP-mutant viruses.

DISCUSSION

293 cell lines that synthesize the artificial transactivator tTA (Gossen and Bujard, 1992) and that efficiently activate expression of a minimal promoter containing repeated tetracycline operators were constructed (Table 1). These cell lines offer a powerful tool for the inducible expression of genes in 293 cells. Using these cell lines, cells that inducibly express high levels of pTP from either a cDNA or a modified genomic construct were constructed and cloned. These cell lines produce biologically active pTP as demonstrated by their ability to efficiently complement the temperature-sensitive pTP mutant virus *sub*100r (Freimuth and Ginsberg, 1986; Schaack *et al.,* 1995a). The level of pTP expression from the pTP cDNA in 293-pTP$_{14}$ cells is substantially greater after induction than the level of expression observed in cell lines that were constructed earlier (Schaack *et al.,* 1995a). The regulated nature of pTP expression in the cell lines described in this study offers increased potential for analysis of pTP function in uninfected cells, such as its interaction with the nuclear matrix (Fredman and Engler, 1993; Angeletti and Engler, 1996), as well as in cells infected with adenovirus strains.

Cell lines derived from 293 cells that express the essential E4 orf6 product have permitted the construction and growth of an adenovirus deleted for both E1 and E4 (Yeh *et al.,* 1996). Similarly, the pTP-expressing cells offer promise for the construction of a pTP deletion-mutant virus (Schaack *et al.,* 1996) as well as deletion mutants lacking both the pTP gene and the E1 region.

Biologically active pTP has been synthesized from baculovirus (Stunnenberg *et al.,* 1988; Zhao *et al.,* 1991) and vaccinia virus (Fredman and Engler, 1993) vectors. The use of viral vectors for expression of pTP offers promise for studies of pTP function in isolation or in reconstituted systems. However, cell lines that express pTP and complement pTP-mutant viruses offer greater hope for genetic analysis of pTP function during the adenovirus infectious cycle and in particular offer promise for the construction of pTP mutants demonstrating a strong phenotype that have proved difficult to construct (Freimuth and Ginsberg, 1986; Roovers *et al.,* 1991, 1993).

The cell lines that inducibly express the highest levels

of pTP from a cDNA and from a modified genomic construct were characterized in detail. The level of pTP expressed from the cDNA in 293-pTP_{14} cells is considerably higher than that expressed from the modified genomic construct in 293-pTP_{40} cells. However, 293-pTP_{40} cells proved slightly and reproducibly more efficient at complementing the pTP defect in the temperature-sensitive mutant *sub*100r in both one-step growth curve and plaquing efficiency assays at the nonpermissive temperature of 39.5° (Tables 2 and 3). In a previous study, the ability of 293-pTP cells to complement *sub*100r for growth at 39.5° was also found to correlate poorly with the level of pTP expressed (Schaack *et al.,* 1995a). The slightly greater ability of 293-pTP_{40} cells than 293-pTP_{14} cells to complement *sub*100r for growth at 39.5° in spite of much reduced pTP expression suggests the possibility that the alternative pTP product, TP*, may be synthesized in 293-pTP_{40} cells and may contribute to viral growth. Alternatively, it may be that overexpression of pTP in a manner not regulated by viral progression through the infectious cycle is detrimental to viral replication or gene expression (pTP expression was induced at the time of the splitting of the cells prior to infection). It is also possible that deleterious effects on the rate of cell growth caused by pTP outweigh its positive effects on viral growth. Additionally, the effects of the different temperatures used in the assays of complementation of *sub*100r on the function of pTP and other viral proteins is not clear. The yield of both wild-type and pTP-mutant viruses in pTP-expressing cells was generally higher at 39.5° than at 32° (Table 3), suggesting the possibility that pTP supplied *in trans* in the cell lines may exhibit some temperature dependence for function. Finally, it is important to note that *sub*100r expresses pTP and maintains a low level of pTP activity at 39.5°. The mutant pTP that is expressed may cooperate or interfere with the wild-type pTP supplied by the host cell. Thus, the most direct assay of pTP function in the 293-pTP cells will involve the use of a virus deleted for pTP expression.

Earlier attempts to express adenovirus DNA pol in mammalian cell lines did not prove successful. However, since DNA pol forms a tight heterodimer with pTP, it was considered possible that expression of both pTP and DNA pol within the same cells would lead to tighter regulation of DNA pol activity and might permit its inducible expression. 293-tTA cells transiently transfected with the plasmid 10-3_{neo}pTP/Pol synthesized both pTP and DNA pol mRNAs as indicated by RNase protection analysis (data not shown). Characterization of the ability of 293-tTA cells stably transfected with 10-3_{neo}pTP/Pol to complement the DNA pol temperature-sensitive mutant *ts*149 at 39.5° demonstrated that little, if any, DNA pol activity was present. Alternative methods of expressing DNA pol in stable cell lines are being examined.

293-pTP_{40} cells have the potential to express the alternative form of pTP mRNA, termed TP* mRNA. TP* protein does not appear to be expressed in uninfected 293-pTP cells, since antiserum against an epitope at the C-terminus of pTP reacted only with pTP (data not shown). It is possible that TP* mRNA is spliced into a reading frame that differs from that of pTP. However, only the pTP reading frame remains open for an extended length from the approximate site of the splice acceptor for TP* mRNA. Thus, it is unlikely that a different translational reading frame is used in TP* mRNA. If analyses currently under way demonstrate that TP* mRNA is expressed in infected 293-pTP cells, the function of the putative protein encoded by this alternatively spliced product will be examined.

The cell lines constructed in this study have utilized a rapidly growing 293 strain. While these cell lines are proving useful for both construction of pTP deletion-mutant viruses and analysis of pTP function, slower growing 293 cells that survive longer after reaching confluence offer a better tool for plaquing of adenoviruses. Therefore, 293 cells from the American Type Culture Collection that grow more slowly have been stably transfected with the tTA gene. An appropriate cell line has been stably transfected with the pTP-encoding plasmid DNAs. The resultant cell lines should offer more powerful tools for use for the construction of pTP-mutant adenoviruses.

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